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Determination of synergic antioxidant interactions of Ellagic acid and chemotherapy drug (Docetaxel and Mitoxantron) combinations

Elajik asit ve kemoterapi ilaç (Dosetaksel ve Mitoksantron) kombinasyonlarının sinerjik antioksidan etkileşimlerinin belirlenmesi

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Abstract

Chemotherapy drugs are commonly used in cancer treatment, despite their numerous negative side effects. Today, there are studies to reduce the side effects of these drugs and to increase their benefits by combining them with natural substances. Our study investigated the antioxidant and antiradical activities of Ellagic acid (EA), a natural substance, DOC and MIX, commonly used chemotherapeutics, as well as their combinations (EA+DOC, EA+MIX). For this purpose, the methods of DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activity, Fe^{3+}-Fe^{2+} and Cu^{2+}-Cu^{1+} reduction capacity were used. After the study, the interactions were analysed with the combination index using Compusyn software. Concentrations with maximum synergy in combinations have been identified. For this purpose, firstly, the combination concentrations used in the antioxidant activity methods and the absorbance values were entered into the Compusyn programme. When DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activity results were entered into the programme, more effective synergistic effect was observed for EA+DOC at lower concentrations (7:7µg/mL), (0.125:1µg/mL) respectively. For EA+MIX, no synergistic effect was observed with the application of DPPH scavenging activity results to the programme, while for ABTS$^{\bullet+}$ scavenging activity, more effective synergistic effect was observed at higher concentrations within the applied doses (0.750:6µg/ml). When Fe^{3+}-Fe^{2+} and Cu^{2+}-Cu^{1+} reduction results were applied to the programme; synergistic effect was observed at higher concentrations (17:17µg/mL) for both EA+DOC and EA+MIX and even strong synergistic effect was observed in many of them. The obtained results provide guidance for the use of these combinations in further studies, such as anticancer and enzyme studies.

Keywords: Antioxidant, Chemotherapy, Compusyn, Drug, Synergistic

Öz

Kemoterapi ilaçları kanser tedavisinde yaygın olarak kullanılan ancak birçok negatif yan etkisi bulunan ilaçlardır. Günümüzde bu ilaçların yan etkilerini azaltmak ve doğal maddelerle birleştirerek faydalarını artırmak için çalışmalar bulunmaktadır. Çalışmamızda doğal bir madde olan Elajik asit (EA), yaygın olarak kullanılan kemoterapötikler olan DOC ve MIX ile bunların kombinasyonlarının (EA+DOC, EA+MIX) antioksidan ve antiradikal aktiviteleri araştırılmıştır. Bu amaçla DPPH$^{\bullet}$ ve ABTS$^{\bullet+}$ giderme aktivitesi, Fe^{3+}-Fe^{2+} ve Cu^{2+}-Cu^{1+} indirgeme kapasitesi yöntemleri kullanılmıştır. Çalışma sonrasında etkileşimler CompuSyn yazılımı uygulanarak kombinasyon indeksi ile analiz edilmiştir. Kombinasyonlarda maksimum sinerjik gösteren konsantrasyonlar belirlenmiştir. Bu amaçla öncelikle antioksidan aktivite metotlarında kullanılan kombinasyon konsantrasyonları ve bulunan absorbans değerleri Compusyn programına girilmiştir. DPPH$^{\bullet}$ ve ABTS$^{\bullet+}$ giderme aktivitesi sonuçları programa girildiğinde EA+DOC için sırasıyla daha düşük konsantrasyonlarda (7:7µg/mL), (0,125+1 µg/mL) daha etkin sinerjik etki gözlemlenmiştir. EA+MIX için ise DPPH giderme aktivitesi sonuçlarının programa uygulanmasıyla sinerjik etki gözlemlenmemiş, ABTS$^{\bullet+}$ giderme aktivitesi için ise uygulanan dozlar içinde daha yüksek konsantrasyonlarda daha etkin sinerjik etki gözlemlenmiştir (0.750:6 µg/ml). Fe^{3+}-Fe^{2+} ve Cu^{2+}-Cu^{1+} indirgeme sonuçları programa uygulandığında; hem EA+DOC hemde EA+MIX için daha yüksek konsantrasyonlarda (17:17µg/mL) sinerjik etki gözlenmiş olup hatta birçoğunda güçlü sinerjik etki gözlemlenmiştir. Elde edilen sonuçlar, bu kombinasyonların antikanser ve enzim çalışmaları gibi daha ileri çalışmalarda kullanılması için yol gösterici olmuştur.

***Anahtar kelimeler**: Antioksidan, Kemoterapi, Compusyn, İlaç, Sinerjik*

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1. Introduction

Cancer is the world's second leading cause of morbidity and mortality and is expected to become even more dominant in the future (Durante et al. 2020). It is estimated that cancer will cause 16.4 million deaths worldwide in 2040 (Aziz et al. 2021). One of the most important cancer treatment methods is chemotherapy. Chemotherapy drugs are an important treatment method for patients especially in patients who cannot be treated surgically. They spread throughout the body via the bloodstream, destroying cancer cells and preventing uncontrolled proliferation (Lennan, 2011; Papadakis, 2016). Chemotherapy protocols and schemes subject to change. according to factors such as cancer type, stage and patient condition. Due to these factors, chemotherapy drugs can be used alone or in combination (Ayvat, 2019).

One of the drugs commonly used in chemotherapy is Docetaxel (DOC). Docetaxel is a commonly used chemotherapy drug for treating various solid tumours, such as head and neck cancer, ovarian cancer, breast cancer, metastatic castration-resistant prostate cancer, and non-small cell lung cancer (Razak et al., 2021; Yan et al., 2022). Mitoxantrone (MIX) (1,4-dihydroxy-5,8-bis[2-(2-hydroxyethylamino)ethylamino]anthracene-9,10-dione), a clinically approved broad-spectrum anticancer drug, is mainly used in the treatment of different types of cancer, including breast cancer, leukaemia, lung cancer, lymphoma and prostate cancer (Buczkowski et al., 2019; Granja et al., 2021).

Although chemotherapy drugs are used for the treatment of cancer types as listed above, there are many known side effects in this process. For example, use of the chemotherapeutic drugs Docetaxel and Mitoxantrone can cause hair loss, amenorrhoea, infertility, phlebitis, hypotension, heart attack, oedema, muscle pain, nausea, vomiting, hypersensitivity, decreased appetite, skin toxicity and bone marrow suppression (Fox, 2006; Martinelli et al., 2009; Şimsek, 2022).

For this reason, many studies have emphasized the importance of working with less toxic and more natural substances to improve quality of life, which is reduced by side effects (Lu et al., 2020; Chai et al., 2021). Thus, studies on combinations of drugs and natural substances are increasing.

Phenolic compounds are phenylalanine and tyrosine derivatives with one or more hydroxyl groups directly attached to an aromatic ring. They are found in high amounts especially in fruits and vegetables (Gündoğdu, 2020; Hazafa et al., 2022).

Phenolic compounds are divided into two groups as phenolic acids and flavonoids. They induce pigmentation, reproduction, growth and resistance to pathogens in plants (Hazafa et al., 2022). They attract attention due to their antioxidant, anti-inflammatory, immunomodulatory, antiallergic and anticancer activities (Anantharaju et al., 2016; Kumar & Goel, 2019).

Ellagic acid (2,3,7,8-tetrahydroxybenzopyranol [5,4,3-cde]benzopyran-5,10-dione) (EA) is a natural compound classified as polyphenolic and generally found in fruits such as strawberries, grapes, pomegranates, pomegranates, currants, cranberries, raspberries, walnuts, almonds and hazelnuts (Kaur et al., 2021; Xue et al., 2022).

Ellagic acids have many biological activities, including antimutagenic, antigenotoxic, antibacterial, anti-inflammatory, antidiabetic, antidepressant, antianxiety, cardioprotective, antiapoptotic, anti-tumour, antioxidant, and anticarcinogenic properties (Garcia-Nino & zazueta, 2015; Kaur et al., 2021).

There are studies in the literature that Ellagic acid has antioxidative effect. In 2009, Özyürek et al. used the cuprac method to determine the antioxidative effect of Ellagic acid (Özyürek et al., 2009). Khanduja et al. (2006) and Mehta et al. (2017) was determined the antioxidative activity of Ellagic acid using DPPH radical scavenging activity (Khanduja et al., 2006; Mehta et al., 2017). In another study, Kılıç et al. (2014) investigated the antioxidant activity of Ellagic acid using the ABTS and DPPH radical scavenging methods. The studies suggest that Ellagic acid has antioxidative activity (Kılıç et al., 2014).

Our literature review found no studies on the antioxidant activity of Docetaxel, Mitoxantrone, or combinations of these chemotherapy drugs with Ellagic acid, which are commonly used in chemotherapy. However, there are studies on antioxidant activity or ROS activity in cell line applications.

There are also studies on determining the synergistic effects of different natural substances without cell application. In 2019, Sumaya and Amit conducted a study to determine the activity of combinations of green tea and basil at certain concentrations using various methods such as DPPH and ABTS. They also investigated the synergistic interactions of these combinations using the Compusyn programme. They stated that the combinations of green tea and basil showed significant antioxidant potential and the strongest synergistic effect ratio was 1:1 (Sumaya & Amit, 2019). In another study, antioxidant interactions of black tea and Ocimum gratissimum binary mixture at different ratios were determined by combination index. It was concluded that the 3:1 ratio had the highest radical scavenging capacity in both in vitro and ex vivo analyses among the ratios (Guleria et al. 2022).

Therefore, in our study, the antioxidant and antiradical scavenging activities of Ellagic acid and Docetaxel and Mitoxantrone chemotherapy drugs separately and in combination (EA+DOC, EA+MIX) were determined.

Then, using the Compusyn programme, the concentrations that may show synergistic or antigonastic effects in combinations were investigated. Determination of the synergistic effect according to the combination index results provided guidance for further studies such as anticancer and enzyme activity in which combinations of Ellagic acid and chemotherapy drugs will be used.

2. Material and method

2.1. Chemicals

Trolox, α-Tocoferol, ABTS, DPPH, Ethanol, BHA, BHT, DMPD, TCA, DMSO, $FeCl_3$, $K_2O_8S_2$, CH_3COONH_4, $CuCl_2$, $K_3Fe(CN)_6$, Ellagic acid, Docetaxel and Mitoxantrone were obtained from Sigma Aldrich.

2.2. Antioxidant activity determination methods

2.2.1. $DPPH^{\bullet}$ scavenging activity

DPPH free radical scavenging activity was determined by the Blois method (Blois, 1958). 1 mM solution of $DPPH^{\bullet}$ radical was used. Samples prepared at a concentration of 1 mg/mL were used in the stock solution. Stock solutions of different concentrations of the samples were transferred to tubes and the final volumes were made up to 3 mL with ethanol. Next, tubes containing varying concentrations were each added with 1 mL of the stock $DPPH^{\bullet}$ solution. The samples was incubated in the dark at room temperature for 30 minutes. The measurement of absorbance at 517 nm was taken against ethanol. A control solution consisting of 3 mL of ethanol and 1 mL of $DPPH^{\bullet}$ radical was used.

2.2.2. $ABTS^{\bullet+}$ scavenging activity

ABTS radical scavenging activity was determined according to the study of Re et al. (Re et al., 1999). 7 mM ABTS solution was prepared and a 2.45 nM persulfate solution was added. Thus, $ABTS^{\bullet+}$ radicals were produced. Prior to using the ABTS radical solution, the absorbance of the control solution was adjusted to 0.700±0.025 nm at 734 nm using 0.1 M phosphate buffer with a pH of 7.4. 1 mL $ABTS^{\bullet+}$ radical solution was added into the tubes prepared at concentrations of 1, 2, 3, 4, 5 and 6 µg/mL. The samples was incubated for a duration of 30 minutes. The measurement of absorbance at 734 nm against ethanol was recorded.

2.2.3. Fe^{3+}-Fe^{2+} reduction activity

The reduction activity of Fe^{3+}-Fe^{2+} was studied using the method determined by Oyaizu. Stock solutions were prepared at a concentration of 1 mg/mL. Stock solutions prepared at varying concentrations were added to the glass tubes. The final volume was made up to 1 mL with distilled water. 2.5 mL of 0.2 M phosphate buffer with a pH of 6.6 and 1% potassium ferricyanide ($K_3Fe(CN)_6$) were added to each tube. The mixture was incubated at 50°C for 20 minutes. After incubation, 2.5 mL of 10% trichloracetic acid (TCA) solution was added to the reaction mixture. After removing 2.5 mL of the supernatant of the solution, 2.5 mL of distilled water and 0.5 mL of 0.5 mL of 0.1% $FeCl_3$ were added. Absorbance was measured at 700 nm against distilled water. Distilled water was used as a control instead of the sample (Oyaizu, 1986).

2.2.4. Cu^{2+}-Cu^{+} reduction capacity

The reduction activities of Cu^{2+}-Cu^{+} were carried out by making slight modifications to the method used for reducing copper ions. To the tubes containing our samples prepared at different concentrations, we added 25 mL of 0.01 M $CuCl_2$ solution, 0.25 mL of $7.5x10^{-3}$ M ethanolic neocuprin solution, and 0.25 mL of 1 M CH_3COONH_4 buffer solution, respectively. Absorbance values were measured at 450 nm against distilled water after 30 minutes (Apak et al., 2006).

2.3. Compusyn

The Chou-Talalay method is based on the median effect equation, which is derived from the law of mass action. This theorem ensures a common link between single and multiple entities. According to the combination index (CI) theorem, drug combinations are quantitatively defined for additive effect (CI=1), synergistic effect (CI<1) and antagonistic effect (CI>1) (Chou, 2010). This theory also provides algorithms for automatic computer simulation of synergism and/or antagonism at any effect and dose level, as shown in the CI plot and isobologram (Chou, 2006).

3. Results and disscussion

3.1. Antioxidant activity and reducing capacity findings for Ellagic acid, chemotherapy drugs and combinations

Ellagic acid and Mitoxantrone were prepared at a concentration of 1 mg/mL for all methods used to determine antioxidant activity. For the study on $ABTS^{\cdot+}$ radical scavenging activity only, the concentration of Ellagic acid was diluted to 1:8 mg/mL. Since dimethyl sulfoxide (DMSO) will be used as a solvent in subsequent cell culture studies, DMSO was used as solvent in this study. In the study, Ellagic acid EA, Docetaxel DOC, Mitoxantrone MIX, Ellagic acid+Docetaxel EA+DOC, Ellagic acid+Mitoxantrone EA+MIX are given as abbreviations.

3.1.1. $DPPH^{\cdot}$ scavenging activity findings

DPPH scavenging activity at 517 nm was tested for the ellagic acid/chemotherapy combinations (EA, MIX, DOC, EA+DOC, EA+MIX). We drew absorbance graphs corresponding to the concentrations and included error bars on the graph (Figure S1, S2) Finally, separately IC_{50} values were calculated (Table 1). No significant decrease in absorbance with increasing concentration was observed for Docetaxel, and therefore it could not be plotted.

3.1.2. $ABTS^{\cdot+}$ scavenging activity findings

The scavenging activity of ellagic acid/chemotherapy combinations (EA, MIX, DOC, EA+DOC, EA+MIX) on $ABTS^{\bullet+}$ was tested at 734 nm. Absorbance graphs corresponding to the concentrations were drawn and error bars were shown on the graph (Figure S3, S4). IC_{50} values were calculated (Table 1). For Docetaxel, no significant decrease in absorbance was observed with increasing concentration, and therefore, it could not be plotted.

Table 1. $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging activity (IC_{50}) values of the substances and combinations and Fe^{3+}-Fe^{2+} and Cu^{2+}-Cu^{+} reduction force (A) values

Substances and Combinations	$DPPH^{\bullet}$	$ABTS^{\bullet+}$	Fe^{3+}-Fe^{2+}	Cu^{2+}-Cu^{+}
	[IC_{50}](µg/mL)	[IC_{50}](µg/mL)	A±SD	A±SD
EA	3.797	0.524	0.964 ± 0.043	0.455 ± 0.011
DOC	-	-	0.064 ± 0.001	0.154 ± 0.004
MIX	6.556	2.461	0.730 ± 0.067	0.420 ± 0.004

Table 1. Continued

EA+DOC	10.999	4.290	0.514 ± 0.010	0.274 ± 0.016
EA+MIX	6.988	1.804	0.661 ± 0.038	0.303 ± 0.042

3.1.3. Fe^{3+}-Fe^{2+} reduction activity findings

The absorbance of Ellagic acid, chemotherapy drugs, and combinations was measured at 700 nm, and corresponding absorbance graphs were plotted with error bars (Figure S5,S6). The increase in absorbance of Docetaxel with increasing concentration is negligibly small.

3.1.4. Cu^{2+}-Cu^{+} reduction capacity findings

The absorbances of Ellagic acid, chemotherapy drugs, and their combinations were measured at 450 nm. The corresponding absorbance graphs were drawn, and error bars were included (Figure S7, S8). For both Fe^{3+}-Fe^{2+} reduction and Cu^{2+}-Cu^{+} reduction methods were compared with each other by giving the absorbance values of Ellagic acid, chemotherapy drugs and combinations at 5 µg/mL (Table 1).

3.2. Determining synergistic effects of in vitro antioxidant activity

In the in vitro antioxidant methods, compusyn programme was used to determine the combination index (CI). To determine the synergistic effect, we used the CompuSyn programme to draw a fractional effect (Fa) and combination index (CI) graph (Fa-CI). Values below CI<1 on the y-axis indicate a synergistic effect, while values below CI<0.5 indicate a strong synergistic effect. At the same time, a CI>1 was considered antagonistic, while a CI=1 was considered additive.

The names given in the tables are abbreviated as follows.
Combination index: CI, Fractional effect: Fa

3.2.1. $DPPH^{\cdot}$ scavenging activity findings

The results obtained from EA, DOC, and MIX using the $DPPH^{\cdot}$ scavenging method were analysed using the Compusyn programme (Table S1). Fractional effect and combination index values of EA+DOC and EA+MIX obtained as a result of the application (Table 2).

Table 2. Doses of EA+DOC and EA+MIX combinations found according to $DPPH^{\cdot}$ scavenging method and entered into Compusyn programme, Fa and CI values obtained as a result of application

Dose (µg/mL)	**Fa** (EA+DOC)	**CI** (EA+DOC)	**Fa** (EA+MIX)	**CI** (EA+MIX)
2.000	0.744	0.898	0.752	1.36489
6.000	0.389	0.962	0.638	2.78084
10.000	0.219	0.919	0.478	2.90873
14.000	0.080	0.582	0.321	2.54697
18.000	0.061	0.614	0.263	2.68293
22.000	0.053	0.678	0.202	2.57149

For $DPPH^{\cdot}$ scavenging method results; The Compusyn programme's Fa and CI graph was used to evaluate the synergistic effect of the EA+DOC and EA+MIX combination (Figure S9, S10).

3.2.2. $ABTS^{\cdot+}$ scavenging activity results

The results obtained from EA, DOC, and MIX using the $ABTS^{\cdot+}$ scavenging method were applied to the Compusyn program (Table S2). The fractional effect and combination index values of EA+DOC and EA+MIX were obtained as a result of the application (Table 3).

Table 3. Doses of EA+DOC and EA+MIX combinations entered into Compusyn programme according to $ABTS^{\cdot+}$ scavenging method, Fa and CI values obtained as a result of application

Dose (µg/mL)	**Fa** (EA+DOC)	**CI** (EA+DOC)	**Fa** (EA+MIX)	**CI** (EA+DOC)
1.125	0.752	0.712	0.457	0.856
2.250	0.639	0.928	0.262	0.990
3.375	0.432	0.742	0.160	1.003
4.500	0.353	0.802	0.042	0.532
5.625	0.339	0.929	0.004	0.150
6.750	0.323	1.058	0.001	0.076

For the $ABTS^{\cdot+}$ scavenging method results, the synergistic effect of EA+DOC and EA+MIX combination was evaluated according to the Fa and CI graph drawn in Compusyn Programme (Figure S11, S12).

3.2.3. Fe^{3+}-Fe^{2+} reduction activity findings

The results obtained from the EA, DOC, and MIX tests using the Fe^{3+}-Fe^{2+} reduction method were applied to the Compusyn programme (Table S3). The fractional effect and combination index values of EA+DOC and EA+MIX were obtained as a result of the application (Table 4).

Table 4. Doses of EA+DOC and EA+MIX combinations entered into Compusyn programme according to Fe^{3+}-Fe^{2+} reduction method, Fa and CI values obtained as a result of application

Dose (µg/mL)	**Fa** (EA+DOC)	**CI** (EA+DOC)	**Fa** (EA+MIX)	**CI** (EA+MIX)
2.000	0.183	0.671	0.198	1.130
10.000	0.496	1.042	0.638	1.118
18.000	0.659	1.084	0.768	1.197
26.000	0.774	0.879	0.921	0.613
34.000	0.869	0.757	0.999	0.021

For the results of Fe^{3+}-Fe^{2+} reduction method, the synergistic effect of EA+DOC and EA+MIX combination was evaluated according to the Fa and CI graph drawn in Compusyn programme (Figure S13, S14).

3.2.4. Cu^{2+}-Cu^{+} reduction capacity findings

The results obtained from the Cu^{2+}-Cu^{1+} reduction (Cuprak) method for EA, DOC, and MIX were applied to the Compusyn programme (Table S4). The fractional effect and combination index values of EA+DOC and EA+MIX were obtained as a result of the application (Table 5).

Table 5. Doses of EA+DOC and EA+MIX combination according to Cu^{2+}-Cu^{1+} reduction (Cuprak) method entered into Compusyn programme, Fa and CI values obtained as a result of application

Dose (µg/mL)	**Fa** (EA+DOC)	**CI** (EA+DOC)	**Fa** (EA+MIX)	**CI** (EA+MIX)
2.000	0.286	0.521	0.256	1.133
10.000	0.374	0.907	0.414	1.081
18.000	0.422	0.962	0.630	0.290
26.000	0.470	0.832	0.951	0.004
34.000	0.578	0.346	0.999	$3.74x10^{-6}$

For the results of Cu^{2+}-Cu^{1+} reduction method, the synergistic effect of EA+DOC and EA+MIX combination was evaluated according to the Fa and CI graph drawn in Compusyn programme (Figure S15, S16).

The study aimed to determine the antioxidant activities and radical scavenging capacities of drugs used in chemotherapy treatment (DOC and MIX), a natural substance EA, and their combinations (EA+DOC, EA+MIX) at different concentrations. Afterwards, the doses used in the antioxidant study were entered into the Compusn programme to examine the synergistic effects of the combinations.

The method used in our study to determine $DPPH^{\cdot}$ scavenging activity is an antioxidant method that is simple, rapid, sensitive, and reproducible. In this study, we calculated the IC_{50} values for the DPPH free radical scavenging activities of EA (1-11 µg/mL), MIX (1-11 µg/mL), EA+MIX (2-22 µg/mL), and EA+DOC (2-22 µg/mL) combinations. The results showed that the DPPH scavenging activities were in the order of EA>MIX>EA+MIX>EA+DOC (Table 1).

The $ABTS^{\cdot+}$ radical scavenging method is another technique used to determine antioxidative activity. In this study, we calculated the IC_{50} values for $ABTS^{\cdot+}$ scavenging activity of various combinations: EA (1-6 µg/mL), MIX (1-6 µg/mL), EA+MIX (1.125-6.750 µg/mL), and EA+DOC (1.125-6.750 µg/mL). The order of potency was found to be EA>EA+MIX>MIX>EA+DOC when compared with each other.

Since no regular absorbance decrease was observed for DOC in both radical scavenging methods, it could not be graphed and no IC_{50} value could be calculated.

One of the two radical reduction methods used in the study is the reduction capacity of ferric ions (Fe^{3+}-Fe^{2+}) by mild modification of Oyaizu. This method involves measuring $Fe[(CN)_6]^{3+}$ through the reduction of $Fe[(CN)_6]^{3+}$ to $Fe[(CN)_6]^{2+}$. This leads to the formation of $Fe_4[Fe(CN)_6]^3$, which is a Prussian blue coloured complex (Gülçin, 2012). In our study, EA (1-17 µg/mL), MIX (1-17 µg/mL), DOC (1-17 µg/mL) and EA+ DOC (2-34 µg/mL), EA+MIX (2-34 µg/mL). When the absorbance values of combinations measured at 5 µg/mL concentration for Fe^{3+}-Fe^{2+} reduction capacity were compared with each other, it was determined that EA>MIX>EA+MIX>EA+MIX>EA+DOC>DOC.

Another reduction method developed by Apak et al. is based on the reduction of Cupric Ions (Cu^{+2}) to cuprous ions by reducing agents or antioxidants in the presence of Neocuproine. In our study, EA (1-17 µg/mL), MIX (1-17 µg/mL), DOC (1-17 µg/mL) and EA+DOC (2-34 µg/mL), EA+MIX (2-34 µg/mL). When the absorbance values measured for Cu^{2+}-Cu^{1+} reduction capacities of combinations for 5 µg/mL concentration were compared with each other, it was determined that EA>MIX>EA+MIX>EA+MIX>EA+DOC>DOC.

In literature studies, Kılıç et al. (2014) conducted a study on the $ABTS^{\bullet+}$ radical scavenging activity of Ellagic acid. The results showed that at a concentration of 20 µg/mL, the activity was 93.9% (Kılıç et al., 2014). Kannan and Quine (2012) conducted a study on the reduction capacity of Ellagic acid for Fe^{3+}-Fe^{2+} at concentrations of 10-50 µM. The absorbance values measured were found to be between 0.05-0.25 (Kannan & Quine, 2012).

When we evaluate the findings obtained from antioxidant methods in our study in general, it is seen that EA, EA and chemotherapy drug combinations have antioxidant activity and radical reduction capacity. Dosetaxel and Mitoxantrone chemotherapy drugs and their combinations with Ellagic acid could not be compared since there were no studies on the antioxidant activities of these drugs in the literature searches. In the study observed that the combination of Ellagic acid and Docetaxel exhibited significantly higher antioxidant activity than Docetaxel alone in both radical scavenging and reduction methods. The fact that the combinations of Ellagic acid and Mitoxantrone showed similar activity with Mitoxantrone is thought to be due to the high antioxidant activity of Mitoxantrone due to the OH groups in its chemical structure. The role of OH groups in antioxidant activity is known from the studies (Sarıkaya et al., 2010; Gülçin, 2012).

Since the antioxidant activity of the combinations was not a sufficient criterion in the study, the results obtained from the antioxidant study were applied to the Compusyn programme to investigate the synergistic effect of these combinations.

Table 2 and Figure S9 show that in the DPPH$^{\bullet}$ scavenging method, CI<1 was found in six doses of the EA+DOC combination, indicating a synergistic effect. The combination's effective concentration dose was 14 µg/mL (CI: 0.582). According to Table 2 and Figure S10, the EA+MIX combination does not have any value below 1 in the Fa-CI graph. Since CI>1, antagonistic effect was observed at all concentrations.

Table 3 and Figure S11 show that in the ABTS$^{\bullet+}$ scavenging method, CI<1 was found for 5 doses of the EA+DOC combination, indicating a synergistic effect. The effective concentration dose for the combination was found to be 1.125 µg/mL (CI: 0.712). Table 3 and Figure S12 show that in the ABTS$^{\bullet+}$ scavenging method, CI<1 was found in five doses of the EA+MIX combination, indicating a synergistic effect. The effective concentration dose for the combination was found to be 6.750 µg/mL (CI: 0.076). At the same time, the treatment at a dose of 5.625 µg/mL (CI: 0.150) was also found to be effective. It appears that there is a strong synergistic effect at both concentrations.

Table 4 and Figure S13 show that the Fe^{3+}-Fe^{2+} reduction method resulted in a CI<1 for three doses of the EA+DOC combination, indicating a synergistic effect. The effective concentration dose for the combination was found to be 2,000 µg/mL (CI: 0.671). Table 4 and Figure S14 show that the Fe^{3+}-Fe^{2+} reduction method resulted in a synergistic effect for 2 doses of the combination of Ellagic acid and Mitoxantrone, with a CI<1. The effective concentration dose for the combination was found to be 34,000 µg/mL (CI: 0.021). A strong synergistic effect was observed for this dose.

According to Table 5 and Figure S15, the Cu^{2+}-Cu^{1+} reduction (Cuprak) method showed a CI<1 for 5 doses in the EA+DOC combination, indicating a synergistic effect. The effective concentration dose for the combination was found to be 34,000 µg/mL, resulting in a strong synergistic effect (CI: 0.346). According to Table 5 and Figure S16, for Cu^{2+}-Cu^{1+} reduction (Cuprak) method; CI<1 was found for 3 doses in EA+ MIX combination. The effective concentration dose for the combination was determined to be 34,000 µg/mL, exhibiting a strong synergistic effect (CI: $3.74.10^{-6}$). At the same time, it is noted that the doses of 18,000 µg/mL (CI: 0.290) and 26,000 µg/mL (CI: 0.004) are also highly effective and exhibit a strong synergistic effect.

Although there are no studies on the combinations used in our study, there are different studies in which combinations of natural substances are formed to determine the synergistic effect. In a study, synergistic antioxidant effects of different phenolic acids and flavonoids were investigated using FRAP method. The study found that the combination of Gallic acid and Caffeic acid demonstrated a significant synergistic effect (137.8%), while the other combinations were less effective (Hajimehdipoor et al. 2014). In a separate study, the researchers investigated the impact of certain antioxidants on the stability of soybean oil. They found that a combination of tetrabutyl hydroquinone and butylated hydroxyanisole (in a 2:1 ratio) had the strongest synergistic effect and antioxidant activity (Guzman et. al. 2019). In their study conducted in 2019, Sumaya and Amit determined the activity of combinations of green tea and basil at certain concentrations by various methods such as DPPH and ABTS and investigated their synergistic interactions using compusyn programme. Green tea and basil combinations showed significant antioxidant potential and the strongest synergistic effect ratio was 1:1 (Sumaya & Amit 2019).

When evaluating our study, we applied the concentrations used in the DPPH and ABTS scavenging activity methods to the Compusyn programme. For EA+DOC, a more effective synergistic effect was observed at lower concentrations (7:7 µg/mL) and (0.125+1 µg/mL), respectively. For EA+MIX, no synergistic effect was observed for DPPH scavenging activity, but an antagonistic effect was observed. However, for EA+MIX, a more effective synergistic effect was observed for ABTS scavenging activity at higher concentrations (0.750:6 µg/mL). In the reduction methods, synergistic effect was observed at higher concentrations (17:17 µg/mL) at the doses applied for both EA+DOC and EA+MIX and even strong synergistic effect was observed in many of them.

4. Conclusions

Currently, natural substances have been shown to prevent various diseases, including cancer, neurodegenerative and cardiovascular disorders, as well as ageing. As a result, there has been a growing number of studies conducted on these substances, both individually and in combination with other substances. In particular, studies on anticancer treatments that reduce the damage caused by chemotherapy drugs by

combining them with natural products or eliminating side effects, as well as studies on enzymes used as disease markers, are of particular interest. In our study, the antioxidant activities and synergies of EA+DOC and EA+MIX combinations, which we could not provide information about the combination effect as a result of the literature review, were primarily examined. The results of Compusyn indicate a synergistic effect in all methods and combinations, except for the EA+MIX combination in the DPPH scavenging method. Concentrations with a strong synergistic effect were also identified. The results obtained suggest that it would be useful to observe the effects of these combinations in further studies such as cell studies and enzyme studies.

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Author contribution

C. Zehiroğlu carried out experimental studies. C. Zehiroğlu and S. B. Öztürk Sarıkaya worked together on the sections related to literature research, graphic drawings and discussion.

Declaration of ethical code

The authors of this article declare that the materials and methods used in this study do not require ethics committee approval and/or legal-special permission.

Conflicts of interest

The authors declare that they have no conflict of interest.

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SUPPLAMENTARY MATERIAL

Figures

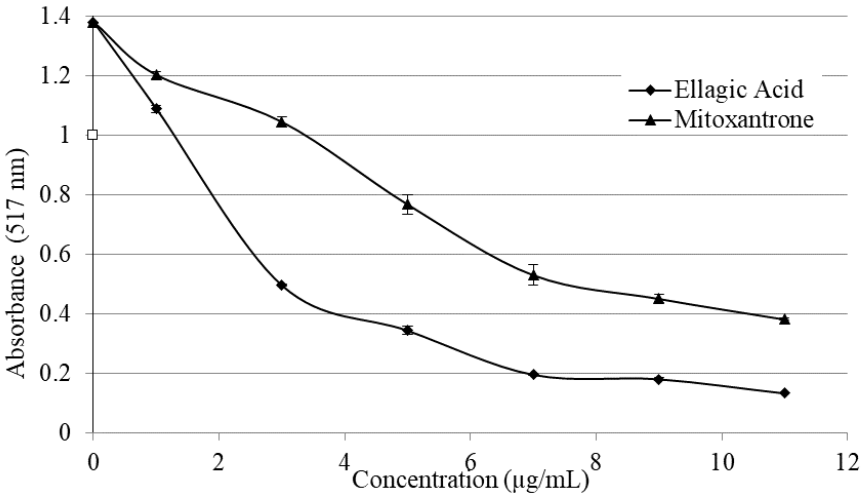


Figure S1. Graph of DPPH· scavenging activity of EA and MIX substances (1-11 µg/mL)

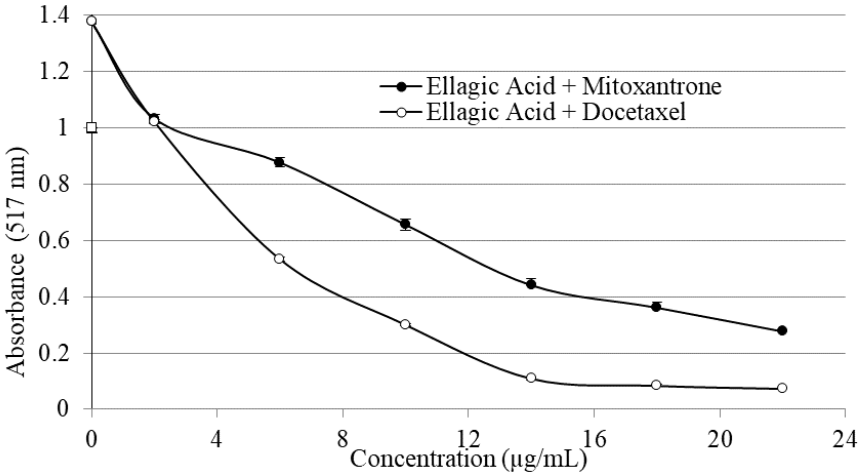


Figure S2. DPPH· scavenging activity graph of combinations (EA+MIX and EA+DOC) (2-22 µg/mL)

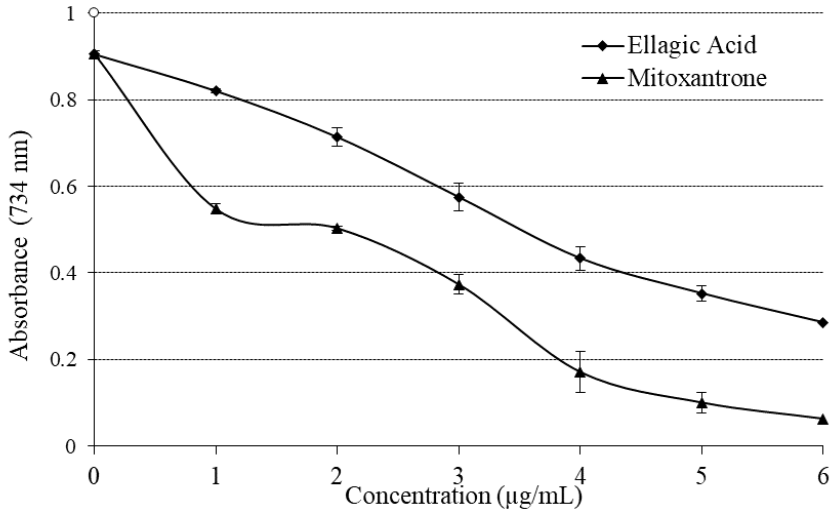


Figure S3. $ABTS^{\cdot+}$ scavenging activity graph of EA and MIX substances (1-6 µg/mL)

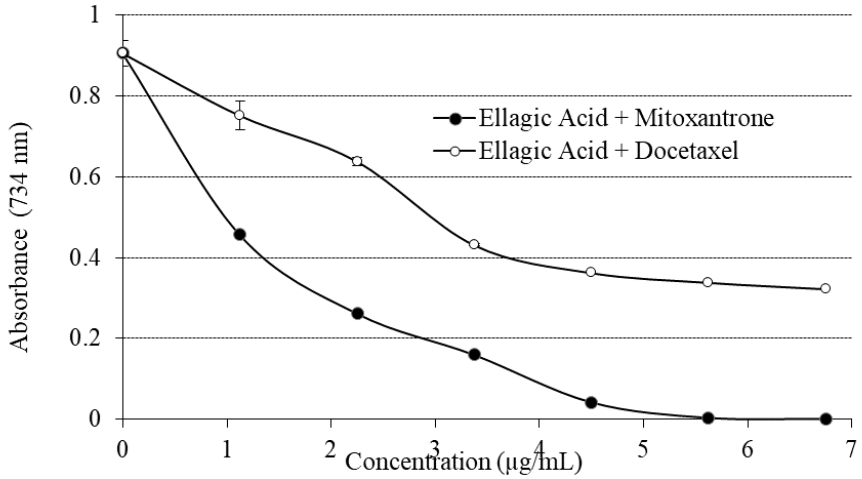


Figure S4. $ABTS^{\cdot+}$ scavenging activity graph of combinations (EA+MIX and EA+DOC) (1.125-6.750 µg/mL)

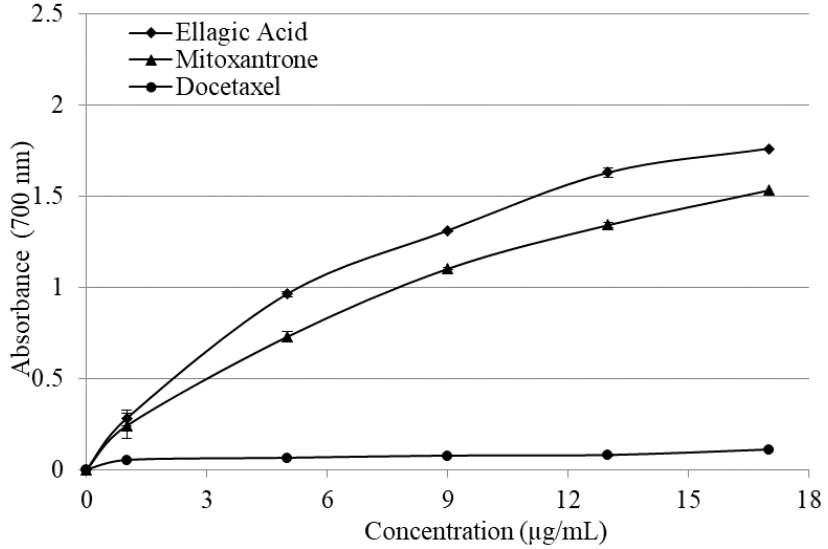


Figure S5. Fe^{3+}- Fe^{2+} reduction capacity graph of EA, MIX and DOC (1-17 µg/mL)

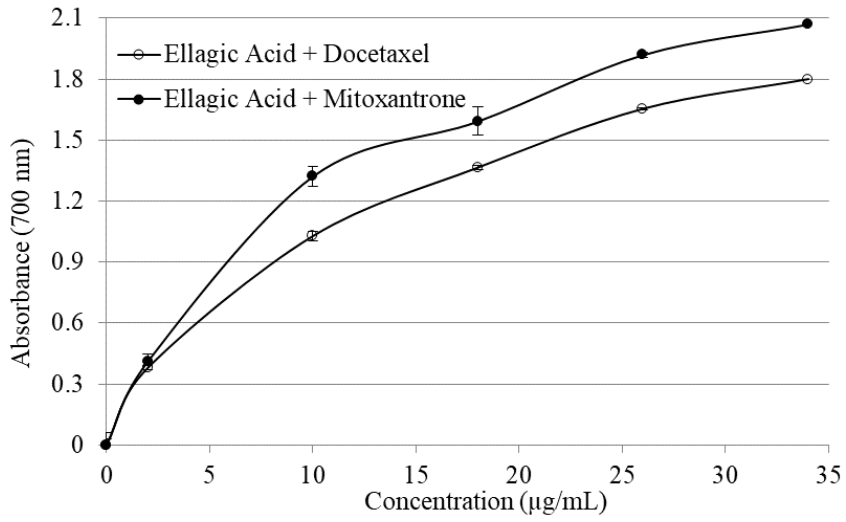


Figure S6. Fe^{3+}- Fe^{2+} reduction capacity graph of combinations (EA+DOC and EA+MIX) (2-34 µg/mL)

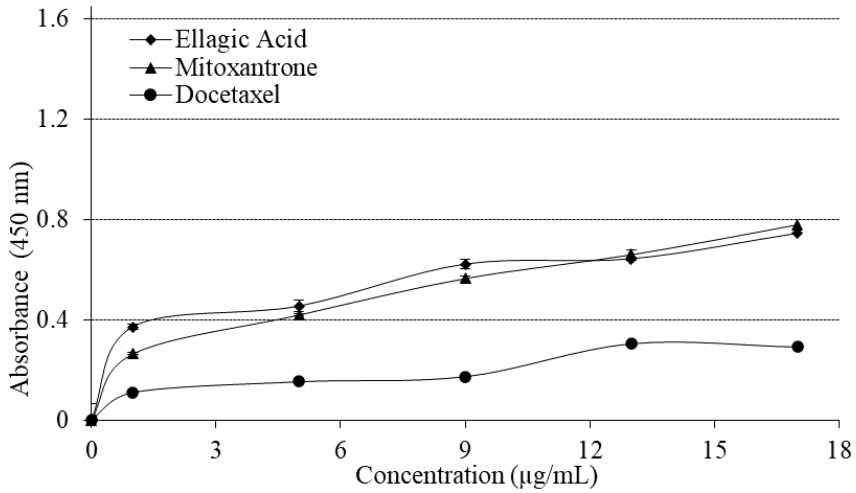


Figure S7. Graph of Cu^{2+} Cu^{1+} reduction capacity of EA, MIX and DOC (1-17 µg/mL)

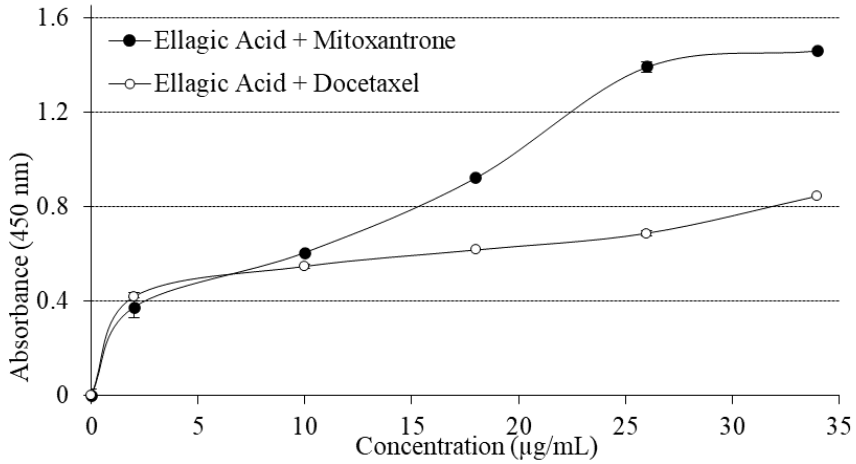


Figure S8. Cu^{2+}-Cu^{1+} reduction capacity graph of combinations (EA+DOC and EA+MIX) (2-34 µg/mL)

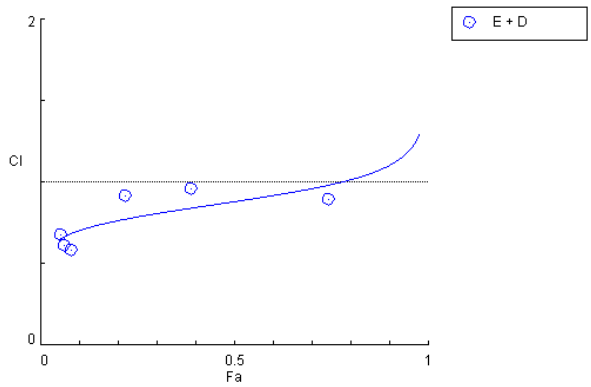


Figure S9. FA-CI graph drawn for EA+ DOC

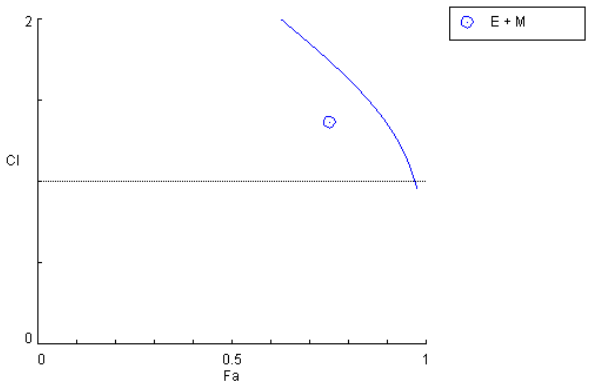


Figure S10. FA-CI graph drawn for EA+ MIX

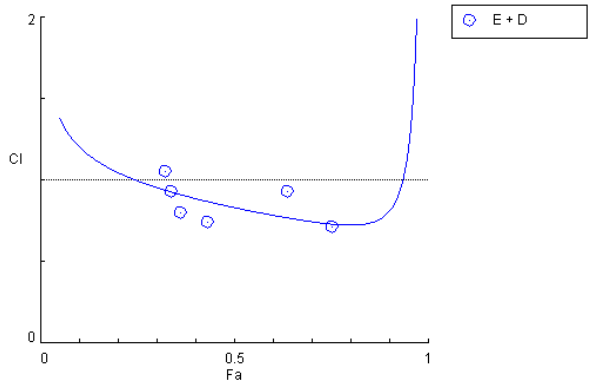


Figure S11. FA-CI graph drawn for EA+ DOC

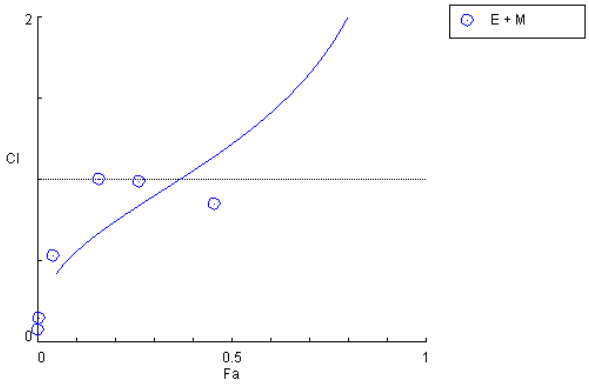


Figure S12. FA-CI graph drawn for EA+ MIX

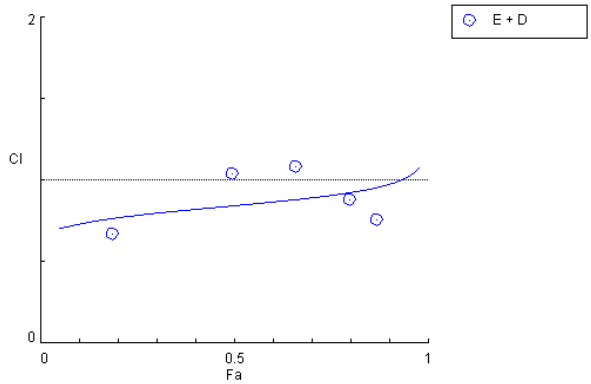
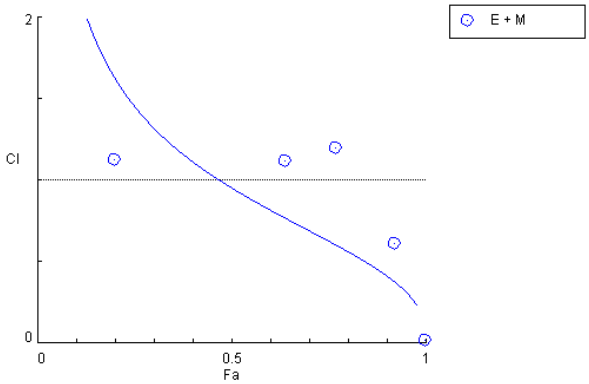


Figure S13. FA-CI graph drawn for EA+ DOC



FigureS14. FA-CI graph drawn for EA+ MIX

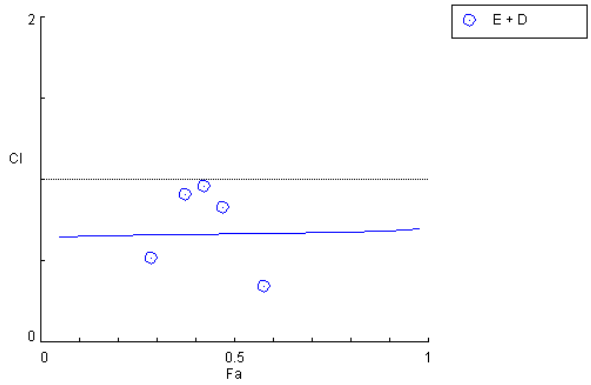


Figure S15. FA-CI graph drawn for EA+ DOC

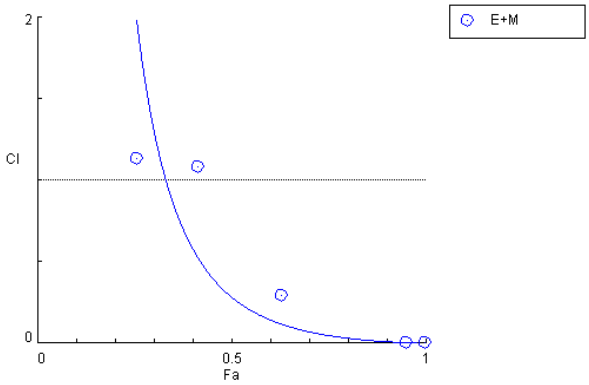


Figure S16. FA-CI graph drawn for EA+ MIX

Tables

Table S1. Dose and absorbance values of EA, DOC and MIX according to $DPPH^{\cdot}$ removal method and entered into Compusyn programme

EA Dose (µg/mL)	EA Absorbance	DOC Dose (µg/mL)	DOC Absorbance	MIX Dose (µg/mL)	MIX Absorbance
1.000	0.791	1.000	0.999	1.000	0.873
3.000	0.359	3.000	0.993	3.000	0.759
5.000	0.249	5.000	0.987	5.000	0. 558
7.000	0.142	7.000	0.995	7.000	0.385
9.000	0.131	9.000	0.995	9.000	0.327
11.000	0.096	11.000	0.992	11.000	0.272

Table S2. Dose and absorbance values of EA, DOC and MIX according to $ABTS^{\cdot+}$ removal method and entered into Compusyn programme

EA Dose (µg/mL)	EA Absorbance	DOC Dose (µg/mL)	DOC Absorbance	MIX Dose (µg/mL)	MIX Absorbance
0.125	0.821	1.000	0.903	1.000	0.548
0.250	0.714	2.000	0.900	2.000	0.504
0.375	0.575	3.000	0.890	3.000	0.374
0.500	0.434	4.000	0.872	4.000	0.171
0.625	0.353	5.000	0.823	5.000	0.101
0.750	0.285	6.000	0.816	6.000	0.063

Table S3. Dose and absorbance values of EA, DOC and MIX according to Fe^{3+}- Fe^{2+} reduction method and entered into Compusyn programme

EA Dose (µg/mL)	EA Absorbance	DOC Dose (µg/mL)	DOC Absorbance	MIX Dose (µg/mL)	MIX Absorbance
1.000	0.136	1.000	0.025	1.000	0.116
5.000	0.465	5.000	0.031	5.000	0.352
9.000	0.632	9.000	0.037	9.000	0.532
13.000	0.786	13.000	0.038	13.000	0.648
17.000	0.850	17.000	0.053	17.000	0.837

Table S4. Dose and absorbance values of EA, DOC and MIX according to Cu^{2+}-Cu^{1+} reduction (Cuprak) method entered into Compusyn programme

EA Dose (µg/mL)	EA Absorbance	DOC Dose (µg/mL)	DOC Absorbance	MIX Dose (µg/mL)	MIX Absorbance
1.000	0.254	1.000	0.076	1.000	0.181
5.000	0.311	5.000	0.105	5.000	0.288
9.000	0.425	9.000	0.120	9.000	0.387
13.000	0.440	13.000	0.209	13.000	0.452
17.000	0.509	17.000	0.200	17.000	0.534